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A sensitive direct sequencing assay based on nested PCR for the detection of HBV polymerase and surface glycoprotein mutations

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A B S T R A C T

Drug resistance is a crucial problem emerging frequently during treatment of hepatitis B, resulting in treatment failure and progression of liver damage. A direct sequencing method based on a nested PCR was established to detect mutations in samples with low viral load.

Primers were designed to obtain an amplicon encompassing the A, B, C, D and E functional domains of HBV polymerase. Fifty-five samples were tested, containing HBV DNA ranging from 19 to 1700 IU/mL. Sixteen samples were also tested by the commercially available assay INNO-LiPA HBV DR v2.

Sequencing was successful for all samples, and mutations were detected in 24/55 (43.6%). When used in parallel with DR v2, concordant results were found in 8/16 samples. In the eight discordant cases, four were resolved by sequencing and not by DR v2, and four had differences in the mutation patterns. Direct sequencing was able to show *pol* mutations not revealed by DR v2, such as rtV214A, rtQ215H/S, and rtM250V. Genotype and *env* variations were also established.

This highly sensitive sequencing protocol, providing valuable sequencing data from samples with a low viral load, is suitable for detection of mutations at the very early signs of failure of treatment, thereby allowing to maximize the success of early treatment changes.

1. Introduction

It is estimated that between 350 and 400 million people are infected chronically with HBV. Chronic HBV infection leads to the development of late complications, such as cirrhosis and hepatocellular carcinoma. Treatment regimens to control HBV replication are effective in reducing the extent of liver damage (Yuan and Lee, 2007). The U.S. Food and Drug Administration approved two types of drugs for the treatment of chronic HBV infection, i.e. immunomodulatory agents (interferon-alpha and peg-interferon) and nucleos(t)ide analogues, which inhibit HBV reverse transcriptase activity, such as lamivudine (LAM), entecavir (ETV), telbivudine (TBV), adefovir (ADV), and tenofovir (TDF). New analogues are under development or in advanced clinical trials, such as emtricitabine (FTC), and clevudine (L-FMAU). However, prolonged therapy with nucleos(t)ide analogs often results in the selection of mutations in the target gene, which confer drug resistance and treatment failure (Pawlotsky et al., 2008). Thus, early detection of antiviral resistant mutants is of clinical importance for choosing new treatment strategies.

To date, the method used most commonly for detecting drug resistance mutations is direct sequencing of the viral *pol* gene after amplification of a selected fragment, using the polymerase chain reaction (PCR). This approach allows the identification of all substitutions, including primary, compensatory, and novel mutations, and the contemporary determination of viral genotype. However, direct sequence-based methods are only capable of detecting mutations present in viral quasispecies with a prevalence ≥20% of the total HBV population (Lok et al., 2007), and primer design, nucleic extraction protocols and PCR conditions affect strongly the ability to generate PCR amplicons in samples with low HBV DNA concentrations (Gintowt et al., 2005).

The only commercially available sequence-based HBV genotypic resistance test, i.e. Trugene HBV Genotyping kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY), is reported to be successful rarely on samples with HBV DNA values below 1000 IU/mL, and the DNA extraction method is known to affect the efficiency of the test (Kessler et al., 2003; Gintowt et al., 2005).

A number of other techniques are available for detecting drug resistance mutations of HBV, such as restriction fragment length/mass polymorphism (Jardi et al., 1999; Sablon and Shapiro, 2004; Hosseini et al., 2006), oligonucleotide microarray and gene

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chip technology (Li et al., 2005; Tang et al., 2007), mutation-specific real-time PCR (Wang et al., 2006) or selective real-time PCR (Lupo et al., 2008), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Kim et al., 2005) as well as pyrosequencing (Ijaz et al., 2008; Solmone et al., 2008), most of which are focussed mainly on LAM resistance mutations (Yeon, 2008). The commercially available test used most frequently is INNO-LiPA HBV DR v2 (DR v2, Innogenetics NV, Ghent, Belgium), based on reverse hybridization technology. This assay, based on a single round PCR, employs a series of short membrane-bound oligonucleotide probes to detect single mismatches in the target region. It has an absolute sensitivity of 172 IU/mL and is able to identify HBV mutants with a prevalence as low as 5% of the total HBV quasispecies, but like all sequence-specific methods has the disadvantage of detecting only known mutations, and periodical update is necessary when new antiviral resistances are identified. A new version of the assay, i.e. DR v3 which addresses the entecavir-related mutations has been established recently although it is not yet employed widely for routine use (Degertekin et al., 2009). Another potential drawback of reverse hybridization technology is that false-negative and/or indeterminate results can occur (Lok et al., 2002).

With the aim of maximizing the advantages of using direct sequencing, while preserving the ability to obtain sequencing data in samples with low HBV DNA concentrations, a nested PCR was designed for the amplification step. The results indicate that the new method described allows successful sequencing from samples with viral loads as low as 19 IU/mL, and is therefore suitable for the detection of mutations at the very early signs of treatment failure; in addition, due to the length of the amplified gene fragment, this method is suitable for the identification of all known HBV drug resistance mutations, as well as those that are novel, and require further investigations to determine their significance.

2. Patients and methods

From January 2006 to February 2008, 317 consecutive patients with chronic hepatitis B, attending the outpatient laboratory service at the National Institute for Infectious Diseases, INMI Lazzaro Spallanzani, Rome, were tested for genotypic HBV drug resistance. With the aim of evaluating the sequencing method performances at low viral loads, patients with HBV DNA loads <2000 IU/mL ($n = 55$) were enrolled.

HBV DNA levels were evaluated by TaqMan HBV (Roche Molecular Systems, Inc., Pleasanton, CA; dynamic range: 12–110,000,000 IU/mL). Serum HBV DNA was extracted by QIAamp Blood kit (Qiagen, Chatsworth, CA, USA) using the automated BioRobot MDx Workstation (MDx, Qiagen).

The PCR amplicons to be sequenced were obtained by nested PCR. The primer sequences were as follows: forward POLHB1F (5′-CCTGCTGGTGGCTCCAGTT-3′, nt 56–74 of HBV genome and reverse POLHB2R (5′-CRTCAGCAAACACTTGRC-3′ nt 1175–1192) for the first round, and forward POLHB3F (5′-CTCGTGGTGGACTTCTCTC-3′, nt 253–271) and reverse POLHB4R (5′-GCAAANCCCMAAAGRCCCAC-3′, nt 1000–1019) for the second round. Amplification conditions for both the first and the second rounds were: denaturation 15 min at 94 °C, then 40 cycles of 1 min at 94 °C, 1 min at 58 °C, 2 min at 72 °C. A final cycle with an elongation step of 10 min at 72 °C was included at the end. The PCR product was purified by QIA quick PCR purification kit (Qiagen).

Sequencing was performed on the automated ABI Prism 3100 instrument, by using BigDye terminator cycle sequencing kit (Applied Biosystem, Warrington, UK). The primers used for the sequencing reaction were the same as those used for the second round of PCR; the sequencing cycling conditions were denaturation 2 min at 95 °C, then 25 cycles of 10 s at 96 °C, 5 min at 55 °C, 3 min at 60 °C.

To define HBV genotype, the *pol* sequences obtained by sequencing were compared to GenBank reference sequences by BLAST search analysis.

On samples with sufficient residual volume ($n = 16$), the genotypic resistance pattern was also determined by using INNO-LiPA HBV DR v2 (Innogenetics NV, Ghent, Belgium) according to the manufacturer's instructions.

3. Results

3.1. "Absolute" and "relative" sensitivity

The percentage of samples with successful sequencing results was 100%: 24 samples (43.6%) showed mutations in the HBV polymerase gene (Table 1), while 31 carried wild type sequences (not shown in the table).

Overall, the direct sequencing detected many mutations described previously, associated to LAM, ADV and ETV resistance. In addition, this method identified novel substitutions at 12 different rt codons (103, 145, 176, 182, 187, 224, 235, 252, 260, 263, 267, 271) (Table 1) which require further investigations in order to assess their functional significance and possible interaction with other mutations.

To establish the absolute sensitivity of the test, triplicate assays using serial dilutions (from 1553 to12 IU/mL) of a sample with known mutations (L180M + M204V) were performed. The results, shown in Table 2, indicate that direct sequencing identified the L180M + M204V mutations in all triplicates of the entire dilution series, i.e. from 1553 to12 IU/mL. The high sensitivity observed in the dilution experiments was consistent with the results obtained with undiluted patient samples. Seven out of 7 samples from patients with HBV DNA values <100 IU/mL, i.e. patients 1–5 and 25 (Tables 1 and 3, respectively, see below) and patient 40 (not shown), were sequenced successfully.

To establish the capacity of this direct sequencing method to detect a mixture of variants, artificial mixtures containing different percentages of wild type and mutant sequences were tested. In particular, two serum samples with known mutational patterns (one viral genome harboured the wild type sequence, and the other carried L180M + M204V mutations) were mixed in different proportions, from 10% to 90%. The results indicated that the lowest level at which the assay can detect mixtures of mutant and wild type virus is around 20% (not shown).

3.2. Mutations in the overlapping env ORF

Since the genome of HBV presents overlapping *pol* and *env* open reading frames (ORF), we analysed the alternative translation frame of nucleotide sequences obtained by direct sequencing to identify the possible *env* mutation patterns in the 24 patients showing *pol* mutations (Table 1). As expected, all the *env* mutations usually associated to *pol* mutations were identified, such as sE164D, sI195M, sL192F, sW196L corresponding to rtV173L, rtM204V, rtA200V, and rtM204I, respectively, as well as some novel mutations (Table 1). In addition, in a patient with HBV DNA of 1260 IU/mL, treated with LAM for 2 years, the recently described mutation rtA181T (Warner and Locarnini, 2008) corresponding to a stop codon in HBsAg (sW172stop) was detected by the direct sequencing method.

3.3. HBV mutation pattern resulting from direct sequencing and INNO-LiPA HBV DR v2

Table 2 shows the DR v2 results obtained with the dilution series tested in parallel with direct sequencing. By using DR v2, the M204V